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Pollen-Mediated Gene Flow from Kentucky Bluegrass under Cultivated Field Conditions

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ABSTRACT

Kentucky bluegrass (*Poa pratensis* L.), one of the most commonly grown turfgrasses in temperate regions, is being developed for possible commercial release with transgenic traits. The use of this technology raises risk assessment questions because *P. pratensis* is perennial, often apomictic, competitive in many habitats, and hybridizes with other *Poa*. To further understand the potential environmental impact of a transgenic *P. pratensis*, we measured intra- and interspecific pollen-mediated gene flow in field conditions from *P. pratensis* to other *Poa*. We used a wagon-wheel design with a glyphosate (*N*-phosphono methyl-glycine) resistant *P. pratensis* as a pollen donor and a pollen receptor plot at 0 m and plots at 13 and 53 m along six equally spaced vectors. Each receptor plot included accessions from 25 *Poa* species. Seedlings from the receptor plants were screened for resistance to glyphosate and potential hybrids verified by PCR and genomic fingerprinting. Hybrids were found with *P. arachnifera* Torrey, *P. interior* Rydb., *P. pratensis* × *P. secunda* J. Presl, and three other *P. pratensis* entries, but did not occur with *P. annua* L., *P. palustris* L., *P. trivialis* L., or *P. compressa* L., among other species. Overall hybrid frequency was 0.048% and hybrid frequency at the 0-m distance was 0.53%. While apomixis in receptor plants and pollen competition likely reduced the number of hybrids, gene flow did occur but at low frequency and over short distances.

KENTUCKY BLUEGRASS is a commonly used turfgrass in the northern USA, Canada, and other temperate regions of the world. *Poa pratensis* is highly apomictic, which helps create the uniform turfs required for recreation, sports, and other intensive applications. Many varieties have apomixis levels greater than 90% (Bashaw and Funk, 1987). *Poa pratensis* is also grown as a forage grass and is of high quality early in spring (Wedin and Huff, 1996) but is not desirable as a hay crop because of its early maturity and low growth, leading to poor forage yield (Stubbendick et al., 1997).

Improvement of *P. pratensis* through intentional breeding is fairly recent compared with most agriculturally important crops. Most early turfgrass breeding efforts (pre 1970s) used collections from populations under various forms of management, by selecting plants showing desirable stress tolerance, growth habit, and appearance. (Bashaw and Funk, 1987; Huff, 2003). Although apomixis simplifies seed increase of *P. pratensis* varieties and provides for uniformity in turf settings, this asexual reproduction complicates traditional breeding processes by reducing hybridization and genetic recombination opportunities (Bashaw and Funk, 1987; Huff, 2003). *Poa pratensis* also displays complex forms of polyploidy that may obscure trait segregation, inheritance, and expression leading to complicated inheritance and trait expression during sexual reproduction (Wendel, 2000). Matzk et al. (2005) have recently reported a model for the control of asexual seed formation in *P. pratensis* involving five genes.

The complex evolution and taxonomy of this genus is largely due to extensive hybridization and introgression among *Poa* species (Bor, 1952; Clausen, 1961). Some of the hybrids with *P. pratensis* reported include *P. alpina* L., *P. arachnifera*, *P. arctica* R. Br., *P. compressa*, *P. longifolia* Trin., *P. nemoralis* L., *P. nervosa* (Hook.) Vasey, *P. palustris*, *P. reflexa* Vasey & Scribn, *P. trivialis*, and *P. secunda* (Knobloch, 1968; Welsh et al., 1987). In addition, apomixis has allowed many *Poa* species to overcome consequences that interspecific hybridization may have on sexual reproduction—most importantly sterility of progeny (Clausen, 1961).

Hybridization among *Poa* species and introgression of genes may be facilitated by the sharing of genomes that species have in common. *Poa pratensis* likely contains the genomes from up to four progenitor species, and these are shared with other allopolyploid *Poa* species (Patterson et al., 2005). Because of the similarities among the species and environments in which they are found, many opportunities appear to exist for gene flow among populations of *Poa* (Johnson and Riordan, 1999) and similar to situations in *Agrostis* (Wipff and Fricker, 2001; Watrud et al., 2004).

Most data on the topic of gene flow are on cultivated crop plants including canola, sunflower (*Helianthus annuus* L.), and corn (*Zea mays* L.) (Stewart, 2004), while a more limited amount of information exists for perennial grasses. Modeling of potential gene flow of wind-pollinated grasses through pollen movement has indicated extensive gene flow in some locations and relatively little in others (Meagher et al., 2003). Field hybridization studies of *Agrostis stolonifera* L. have shown gene flow distances of up to 298 m from a pollen source of 286 plants and extrapolated gene flow through modeling to 1066 m in one study (Wipff and Fricker, 2001). Gene flow was reported from an *A. stolonifera* seed field of approximately 162 ha to a sentinel plant of *Agrostis* at a distance of 21 km (Watrud et al., 2004). Gene flow in perennial ryegrass (*Lolium perenne* L.) was measured at less than 2% at 144 m in a downwind direction from 268 plants as a pollen source (Cunliffe et al., 2004) and less than 1% in tall fescue at 150 m with no gene flow observed at 200 m using 49 plants as a pollen source (Wang et al., 2004).

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A number of turfgrasses have been the focus of transgenic breeding, including tall fescue (*Festuca arundinacea* Schreb.), perennial ryegrass, bermudagrass [*Cynodon dactylon* (L.) Pers.], and creeping bentgrass (Zilinskas and Wang, 2004) because of strong interest in the turfgrass industry for herbicide resistance, pest resistance, and environmental stress tolerance traits (Johnson and Riordan, 1999; Ostermeyer, 2004). Because large amounts of Kentucky bluegrass are used around the world, a transgenic *Poa* may be petitioned for a future release. In order for such a petition to be evaluated properly, risk assessment questions, including the potential for introgression of genes from *P. pratensis* into other species, must be considered to establish an understanding of how such varieties may impact local and regional ecosystems.

Although intraspecific and interspecific hybrids involving *P. pratensis* have been described, quantitative assessments of relative hybridization frequencies under field conditions are lacking. Moreover, the impact of apomixis on pollen-mediated gene flow is not fully understood. Our objective was to quantify intra- and interspecific pollen-mediated gene flow from a *P. pratensis* genotype to other *Poa* species by testing hybridization potential in field conditions using herbicide tolerance as a selectable trait.

MATERIALS AND METHODS

Field Layout and Procedures

To evaluate gene flow frequency and distance from a planting of a *P. pratensis* variety to other *Poa* species, we used a "wagon wheel" design (Fig. S1) established in Cache County near Logan, UT. At the center of the wheel, or hub, a ring of 500 glyphosate resistant *P. pratensis* (BR99-1033) were established as the pollen donor plot. BR99-1033 included a single insertion of the CP4 EPSPS gene that encodes 5-pyruvylshikimate-3-phosphate synthase from *Agrobacterium* spp. strain CP4 as a marker to follow gene movement. This gene confers resistance to glyphosate. To observe hybridization frequencies with a wide range of *Poa* species, we established pollen receptor plots along six equally spaced vectors. These nontransgenic pollen receptors included 39 accessions representing 25 *Poa* species (Table 1). Species tested here were chosen on the basis of natural occurrence (native or naturalized) in the western USA, potential hybrid candidates based on previous reports of *Poa* hybrids, and species that are considered weeds in some agronomic or turfgrass production situations. Two plots were established along each vector and centered at 13 and 58 m from the outside edge of the pollen donor plot. The arrangement was planned to accommodate one vector to be downwind of the pollen donor plot, according to the prevailing SW winds of the area. A receptor plot was also established at the very center, within the ring of pollen donor plants, to create a 0-m

Table 1. *Poa* species entries planted in each of 13 receptor plots.

GRIN PI no.	Name	Reproductive mode	Collection location
PI 372559	*P. alpina*	apomictic & sexual	from Alberta, Canada
	P. annua	sexual	from Canada
	P. annua var. *reptans*	sexual	near Logan, UT
PI 236900	*P. arachnifera*†	sexual	unknown
PI 236901	*P. arctica*	apomictic & sexual	from Canada
PI 371755	*P. brachyanthera* PN-610	sexual‡	from Alaska, USA
PI 182792	*P. compressa*	apomictic & sexual	from Ontario, Canada
PI 243216	*P. compressa*	apomictic & sexual	from Virginia, USA
	P. fendleriana†	sexual	western USA
PI 578808	*P. hybrid* P-14094§	apomictic & sexual	from Washington, USA
PI 578818	*P. hybrid* 'Little Enchantress'§	apomictic & sexual	from Washington, USA
PI 325462	*P. iberica* Fisch. & C. A. Mey. S-301	sexual	from Stavropol, Russian Federation
PI 236909	*P. interior*	sexual	from Canada
PI 284254	*P. iridifolia* Hauman CPI 9724	sexual‡	sexual‡
PI 154883	*P. lanigera* Nees	sexual‡	from Uruguay
PI 380991	*P. longifolia*	sexual‡	from Iran
PI 325464	*P. nemoralis* S-92	apomictic & sexual	from Stavropol, Russian Federation
PI 371759	*P. nemoralis* PN-614	apomictic & sexual	from Alaska, USA
PI 232352	*P. nervosa*	apomictic & sexual	from USA
PI 232351	*P. palustris*	apomictic & sexual	from USA
PI 387934	*P. palustris*	apomictic & sexual	from Canada
PI 317504	*P. pratensis* subsp. *angustifolia*	apomictic & sexual	from Afghanistan
	P. pratensis Coventry	apomictic & sexual	cultivated variety
	P. pratensis Fairfax	apomictic & sexual	cultivated variety
	P. pratensis Kenblue¶	apomictic & sexual	cultivated variety
	P. pratensis Midnight	apomictic & sexual	cultivated variety
	P. pratensis (Rutgers)	apomictic & sexual	breeding line
	P. sandbergii	apomictic & sexual	western USA
PI 504370	*P. secunda* D&S 51	apomictic & sexual	from Oregon, USA
PI 578851	*P. secunda* P-8903	apomictic & sexual	from Washington, USA
PI 206741	*P. siancia*	sexual‡	
PI 369300	*P. sibirica*	sexual‡	from Former Soviet Union
PI 263863	*P. sieberiana* 'Tarndale'	sexual‡	from New Zealand
PI 236922	*P. stenantha*	sexual	from Canada
	P. supina	sexual	cultivated variety
PI 204484	*P. trivialis*	sexual	from Turkey
PI 303062	*P. trivialis* 'Ino Daelmfeldts'	sexual	from Denmark
PI 283962	*P. trivialis* subsp. *sylvicola*	sexual	from Former Soviet Union
PI 289643	*P. trivialis* subsp. *sylvicola* E-11	sexual	from Spain

† Female plant.
‡ Descriptions of this species in the literature did not mention apomixis, therefore reproductive mode is assumed to be sexual recombination.
§ *Poa pratensis* × *P. secunda* hybrid.
¶ The variety Kenblue contained several lines varying in apomixis level; however, all plants in this experiment were clonally propagated from one original parent.

distance and maximize hybridization potential. This created a total of 13 receptor plots. All plants were clonally propagated and planted to the field in September 2001. The glyphosate resistant *P. pratensis* was maintained under USDA-APHIS guidelines for a transgenic field release for research purposes (Notification numbers 01-187-02n and 02-184-01n).

Each receptor plot included three blocks of accessions in a randomized complete block design. Each block contained one plant from each accession, in two rows of 20 plants. Three replications of the blocks within each plot resulted in a total of six rows in each receptor plot. Rows were spaced 1 m apart and plants within rows were spaced 0.5 m apart. The area between the pollen donor and receptor plots was mowed alfalfa (*Medicago sativa* L.) in the south half of the field and bare soil in the north half (Fig. S1). The entire field plot was irrigated during the growing season every 10 to12 d with an overhead center pivot irrigation system; however, some of the *Poa* species entries did not survive this relatively infrequent irrigation schedule. Summer in the Intermountain West is characterized by relatively warm temperatures and a rain-free period between late May and October.

Weather data was recorded at the plot area on 15-min intervals from April through July of 2002 and 2003 with a WatchDog 900ET weather station (Spectrum Technologies, Plainfield, IL). Measurements included wind direction, wind speed, and wind gusts, relative humidity, temperature, and solar radiation.

In 2002 and 2003, all plants were monitored weekly for inflorescence emergence and anthesis from May through early July and at least twice weekly during anthesis of BR99-1033. Seed from the receptor plot was harvested from each plant when mature but before excessive shattering. This occurred from mid-June to early July. Seed from *P. annua* and *P. supina* Schrad. was harvested throughout June and early July because of their indeterminate flowering. Seed from BR99-1033 was harvested in early July of both years. All seed was air dried and cleaned by hand.

Seedling Evaluation

We screened seedlings from the receptor plants for resistance to glyphosate. Seed from the receptor plants were sowed into greenhouse flats containing moist potting mix composed of 50% peat and 50% perlite (by volume), covered lightly, watered, and stratified for at least 2 wk at 4 to 6°C. Control flats were also seeded and evaluated, which included seedlings of glyphosate susceptible *P. pratensis* 'Coventry' and seedlings from BR99-1033. After stratification, the flats of seed were moved to a warm greenhouse 22°C/18°C for germination and growth. When seedlings were at the 1 to 2 leaf stage, they were counted individually, if under 100 seedlings per flat. If more than 100 seedlings germinated per flat, a grid was usually used to count seedlings in a random 10% of the flat and a total number of seedlings in the flat was then estimated. The seedlings were sprayed with a 1% (v/v) glyphosate solution at the 2 leaf stage, and a second spray, at the same concentration, was made 14 d later with a complete kill of susceptible seedlings obtained within 4 wk of the initial spray. Surviving seedlings were transplanted to pots and grown for verification. Flats were monitored every 2 to 3 d for additional germination after the seedling counts. Additional seedling counts and herbicide sprays were conducted if new seedlings were identified.

Hybrid Confirmation

The presence of transgenic DNA in putative hybrids was verified by PCR amplification of the cauliflower mosaic virus 35S-promoter sequences and *Agrobacterium tumefaciens* nopaline synthase-terminator (NOS) sequences, using the 35S-1//35S-2 and NOS-1//NOS-3 primer combinations (Table S1) described by Lin et al. (2001). The identity of the 195 bp 35S.1//35S.2 and 180 bp NOS.1//NOS.3 amplicons from the BR99-1033 genotype was verified by DNA sequencing. Routine screening with this PCR assay was performed by agarose gel electrophoresis using 100-bp ladder size standards, transgenic BR99-1033 positive control, nontransgenic Coventry negative control, and water negative control as references for each set of assays.

To eliminate possible seed contaminations from BR99-1033, the maternal parent identity was verified by sequencing polymorphic chloroplast DNA regions of the *ndhF* gene and/or *trnK-rps16* intergenic spacer. The *ndhF* gene was amplified and directly sequenced using primers (Table S1) described by Olmstead and Sweere (1994). The *trnK-rps16* intergenic spacer was amplified and directly sequenced using the primers (Table S1) described by (Kress et al., 2005).

Data Analysis

Hybrid frequency data were analyzed in comparison to linear, quadratic, logarithmic, and exponential decay function models that mimic gene or pollen flow to describe the relationship of percentage of hybrids with distance from the pollen source. These analyses were done using SigmaPlot 8.0. Wind summaries were made using WindRose version 2005-02-03 (Enviroware, Agrate Brianza, Italy).

RESULTS AND DISCUSSION

Anthesis Summary

Most species in the receptor plots showed distinct and overlapping periods of anthesis with BR99-1033. Exceptions included the two *P. compressa* entries, which flowered after BR99-1033 in both 2002 and 2003. *Poa fendleriana* (Steud.) Vasey was one of the earliest species to flower. Although data showed overlapping flowering, the receptivity of the female *P. fendleriana* was difficult to determine and may not have been receptive as long as indicated. *Poa annua*, *P. supina*, *P. stenantha* Trin., *P. brachyanthera* Hultén, and *P. arctica* flowered over relatively long periods in 2002 but did not survive into 2003. *Poa alpina* and *P. nervosa* flowered early in 2002 and continued to flower throughout nearly all of the observation period; however, both flowered for a much shorter period in 2003. The anthesis period of BR99-1033 was identical to that of *P. pratensis* Coventry, which was expected since BR99-1033 was developed from Coventry. Temporal separation of flowering is an effective method to prevent gene flow between species (Levin and Kerster, 1974), although it appeared that most of the species tested here do flower at approximately the same time, creating the potential for gene flow.

Weather Summary

Wind patterns were variable and distinctly different in each year of the study. In 2002, winds started predominantly from the SE then switched to the SW, then NE, then finally returned to the SW. Very little wind came from the W or NW directions overall. In 2003, wind direction was dominated by NW, S, and SE directions with little from the SW or NE directions. In 2002, average wind speeds were less than 2 m s^{-1} 50% of the time and

15% were above 4 m s^{-1}. In 2003, wind speeds were less than 2 m s^{-1} 55% of the time and 16% above 4 m s^{-1}. Wind gusts in 2002 were 62.4% above 4 m s^{-1} and 12.7% above 8 m s^{-1}. In 2003, 63.4% of the wind gusts were above 4 m s^{-1} and 17.6% above 8 m s^{-1}. All of these wind data were summarized between 3 and 10 am, the typical period of anthesis during the day.

Relative humidity during anthesis in both years was approximately 80 to 90% at 3am, 75 to 95% at 0600 h, then dropping to 35 to 70% by 1000 h. These humidity conditions are similar to those experienced in the bluegrass seed growing regions of Idaho, eastern Oregon, and eastern Washington.

Seedling Screening Results

Overall, very low levels of inter- or intraspecific hybridization were detected in this experiment during the 2 yr of the study. When seedlings from all entries and all the receptor plots are pooled together in both years, a hybrid frequency of 0.048% occurred (Table 2). Hybrids were not observed with *P. fendleriana*, also a female plant, likely because of temporal separation. Hybrid frequency for female plants of *P. arachnifera* was higher than the other species at 3.4%, while the other interspecific hybrid frequencies ranged from 0.0 to 0.196% (Table 2). Hybrids occurred with *P. interior*, *P. pratensis* × *P. secunda*, and three *P. pratensis* entries ['Kenblue', 'Rutgers', and *P. pratensis* subsp. *angustifolia* (L.) Dumort.]. All *P. pratensis* Coventry seedlings (susceptible control seedlings) died and all seedlings from BR99-1033 (resistant control seedlings) survived the glyphosate screening (Table 2).

Surprisingly, hybrids were not detected in our study with *P. pratensis* and *P. secunda*, which has been a frequently reported hybrid (Hiesey and Nobs, 1982; Knobloch, 1968; Welsh et al., 1987). *Poa secunda* is similar genetically to *P. pratensis* (Patterson et al., 2005; Gillsepie and Soreng, 2005). Hybrids were also not detected with *P. trivialis* or *P. annua*. These species are of great interest because movement of genes such as herbicide resistance into either species, both of which are weeds in some situations, would be of great concern to turf managers and seed producers. The lack of hybrids of *P. trivialis* with *P. pratensis*, even with relatively large numbers of seedlings evaluated, does not prove this cross will not occur but does indicate that the occurrence of the hybrid is expected to be relatively rare (Dixon et al., 2005). No hybrids were detected with *P. annua*, but because we only were able to collect seed from this species in only 1 yr, we must be careful making conclusions about this cross. In a later, and similar, study many more *P. annua* seedlings were evaluated and no hybrids were detected (unpublished data).

The chloroplast genome of diploid *P. trivialis* and tetraploid *P. annua* are distinct from *P. pratensis* (Soreng, 1990; Gillespie and Boles, 2001; Patterson et al., 2005) and in examination of two nuclear genes CDO504 and TRX, only one *P. pratensis* TRX sequence showed any affinity to corresponding sequences of *P. trivialis*. This *P. pratensis* TRX sequence displayed significantly more similarity to corresponding *P. secunda* and *P. arid*a sequences, than it did to *P. trivialis* (Patterson et al., 2005). *Poa annua* TRX sequences showed far less affinity with corresponding sequences of *P. pratensis* (Patterson et al., 2005).

Even among the *P. pratensis* receptor plants, gene flow was low. Highly apomictic *Poa* would be expected to experience very low gene flow from another parent, explaining the lack of hybrids from the apomictic 'Fairfax' and Coventry varieties. Apomixis in many *Poa* species may significantly reduce the occurrence of hybrids because of the reduced frequency of sexually produced progeny. The more sexual *P. pratensis* Rutgers line, Kenblue, and 'Midnight' produced hybrids but still at low frequency.

The pollen donor plants (BR99-1033) appeared highly apomictic on the basis of FISSR profiles and uniform morphological characteristics of all 500 plants in the field. Seedlings from BR99-1033 were not evaluated for morphological characteristics, but a very high frequency of apomixis is suggested since all BR99-1033 seedlings survived the herbicide screen (Table 2). Glyphosate resistance in this variety is conferred by a single gene insert which would be expected to be heterozygous and show segregation if genetic recombination had occurred.

Measured hybridization rates using our methods may have been lower than actual hybridization rates. The glyphosate resistant gene construct was inserted into one location in the pollen donor plant polyploid genome and may be heterozygous for the trait; therefore, producing pollen with and without the herbicide resistance gene via meiosis. *Poa pratensis* appears to have up to four, and possibly more, parental genomes based on work by Patterson et al. (2005). However, these data represent an accurate measurement of transgene movement through hybridization into other populations.

Hybrid Confirmation

The NOS and 35S DNA PCR amplification products were consistently detected and clearly visible in the BR99-1033 transgenic pollen donor parents and glyphosate-tolerant progeny (putative hybrids) obtained from nontransgenic receptor plants (Table 2). Faint amplification products have been observed in the negative controls, but these false-positive tests were eliminated in subsequent testing. One of the glyphosate tolerant *P. arachnifera* progeny died before DNA was sampled; however, the other two transgenic *P. arachnifera* progeny were weak and difficult to maintain in our greenhouse environment. Otherwise, these results confirmed the presence of transgenic DNA in all putative hybrids (Table 2).

With one noted exception, the maternally inherited chloroplast DNA *ndhF* and/or *rps16-trnK* sequences of all putative (transgenic) interspecific hybrids analyzed were identical to the nontransgenic maternal receptor genotypes (Table 2) and different from the paternal BR99-1033 genotype. The chloroplast DNA of one glyphosate-tolerant transgenic seedling, allegedly grown from *P. palustris* PI387934 seed, contained the chloroplast *ndhF*-1 and *rps16-trnK*-3 alleles characteristic of the transgenic BR99-1033 genotype. However, the sequences of the

Table 2. Number of seedlings tested for glyphosate tolerance in 2002 and 2003: number of glyphosate tolerant transgenic seedlings confirmed by PCR, percent transmission of transgene, and unique chloroplast DNA marker sequences (GenBank Accession numbers) used for verification of genetic identity for each accession and species summed over all receptor plots.

	Seedlings tested	Transgenic seedlings	% transmitted	*ndhF* identifier	*rps16-trnK* identifier
All species	**342769**	**161**	**0.05**		
P. arachnifera	**88**	**3**	**3.40**	**AY589107**	**DQ389141**
P. alpina	**29**	**0**	**0**	**AY589097**	
P. annua	**2469**	**0**	**0**		
PI 236900	**2227**	**0**	**0**		
var. *reptans*	**242**	**0**	**0**	**AY589095**	
P. arctica	**584**	**0**	**0**	***ndhF*-1†**	
P. brachyanthera	**76**	**0**	**0**		
P. compressa	**13101**	**0**	**0**	**AY589115**	
P. fendleriana	**24**	**0**	**0**	**AY589106**	
***P. secunda* × *pratensis* hybrids**	**19944**	**39**	**0.20**		
PI 578808	**13439**	**27**	**0.20**	***ndhF*-3§**	
PI 578818	**6505**	**12**	**0.18**	***ndhF*-3§**	
P. iberica	**117**	**0**	**0**	***ndhF*-1†**	**DQ389141**
P. interior	**16946**	**7**	**0.04**	***ndhF*-2†**	**DQ389137**
P. iridifolia	**587**	**0**	**0**	***ndhF*-4¶**	
P. lanigera	**8746**	**0**	**0**		
P. longifolia	**9260**	**0**	**0**		
P. nemoralis	**18072**	**0**	**0**		
PI 325464	**16645**	**0**	**0**		
PI 371759	**1427**	**0**	**0**	***ndhF*-2†**	
P. nervosa	**272**	**0**	**0**	**AY589104**	
P. palustris	**3202**	**0**	**0**		
PI 232351	**2144**	**0**	**0**	***ndhF*-2†**	
PI 387934	**1058**	**0**	**0**	***ndhF*-2†**	**DQ389139**
P. pratensis	**84733**	**112**	**0.13**		
PI 317504	**5032**	**7**	**0.14**		**ps16-trnK-4§§ DQ389140¶¶**
Coventry	**10238**	**0**	**0**	***ndhF*-1†**	***rps16-trnK*-3‡‡**
Fairfax	**5000**	**1**	**0.02**	***ndhF*-1†**	***rps16-trnK*-3‡‡**
Kenblue	**18794**	**16**	**0.08**	***ndhF*-1†**	***rps16-trnK*-1# *ps16-trnK*-4§§**
Midnight	**26145**	**12**	**0.05**	***ndhF*-1†**	***rps16-trnK*-1# *rps16-trnK*-2†† *rps16-trnK*-3‡‡**
Rutgers	**19524**	**80**	**0.41**	**ndhF-1†**	***rps16-trnK*-1# *rps16-trnK*-2††**
***P. sandbergii* J. Presl**	**2158**	**0**	**0**	**AY589111**	
P. secunda	**8645**	**0**	**0**		
PI 504370	**3015**	**0**	**0**	**AY589112**	
PI 578851	**5630**	**0**	**0**	***ndhF*-3§**	
***P. sibirica* Roshev.**	**19300**	**0**	**0**		
***P. sieberana* Spreng.**	**15**	**0**	**0**	***ndhF*-4¶**	
***P. sinaica* Steud.**	**21871**	**0**	**0**		
***P. stenantha* Trin.**	**810**	**0**	**0**		
P. supina	**130**	**0**	**0**	**AY589096**	
P. trivialis	**111670**	**0**	**0**		
PI 204484	**31063**	**0**	**0**	**AY589119**	
PI 283962	**36694**	**0**	**0**		
PI 303062	**22009**	**0**	**0**		
PI 289643	**21904**	**0**	**0**		
BR99-1033 (resistant control)	**4148**	**4148**	**100.00**	***ndhF*-1†**	***rps16-trnK*-3‡‡**
Coventry (suscep. control)	**11333**	**0**	**0**	***ndhF*-1†**	***rps16-trnK*-3‡‡**

† Shared *ndhF*-1 sequence includes *P. pratensis* cv. Coventry (AY589100), *P. pratensis* cv. Kenblue (AY589101), *P. artica* (AY589102), *P. iberica* (AY589103), one transgenic *P. pratensis* cv. Fairfax offspring, two transgenic *P. pratensis* cv. Midnight offspring, five transgenic *P. pratensis* Rutgers offspring, and one *P. pratensis* "BR99-1033" transgenic genotype (DQ377142).

‡ Shared *ndhF*-2 sequence includes two *P. interior* (AY589116) receptor genotypes, six transgenic *P. interior* offspring, *P. nemoralis* (AY58911), *P. palustris* PI 232351 (AY589118), and two *P. palustris* PI 387934 genotypes.

§ Shared *ndhF*-3 sequence includes *P. secunda* PI 578851 (AY589113), three PI 57808 *P. secunda* × *P. pratensis* receptor genotypes, 21 transgenic PI 57808 *P. secunda* × *P. pratensis* offspring, one PI 578818 *P. secunda* × *P. pratensis* receptor genotype, and 11 transgenic PI 578818 *P. secunda* × *P. pratensis* offspring.

¶ Shared *ndhF*-4 sequence includes the *P. sieberiana* (AY589105) and *P. iridifolia* (AY589108).

 # Shared *rps16-trnK*-1 sequence includes two *P. pratensis* Rutgers receptor genotypes (DQ377145), 79 transgenic Rutgers offspring, two transgenic *P. pratensis* cv. Kenblue offspring, and one transgenic *P. pratensis* cv. Midnight offspring.

†† Shared *rps16-trnK*-2 sequence includes two *P. pratensis* cv. Midnight receptor genotypes (DQ377146), nine transgenic *P. pratensis* cv. Midnight offspring, and one transgenic *P. pratensis* Rutgers offspring.

‡‡ Shared *rps16-trnK*-3 sequence includes *P. pratensis* cv. Fairfax (DQ377147), 1 transgenic *P. pratensis* cv. Fairfax offspring, *P. pratensis* cv. Coventry (DQ377147), *P. pratensis* 'BR99-1033' (DQ377147), and one transgenic *P. pratensis* cv. Midnight offspring.

§§ Shared *rps16-trnK*-4 sequence includes *P. pratensis* subsp. *angustifolia* receptor genotypes (DQ377148), 5 trangenic *P. pratensis* subsp. *angustifolia* offspring, two *P. pratensis* cv. Kenblue receptor genotypes, and 14 transgenic *P. pratensis* cv. Kenblue offspring.

¶¶ Sequence unique to one putative *P. pratensis* subsp. *angustifolia* × *P. pratensis* BR99-1033 transgenic hybrid and not detected among any of the pollen donor or receptor parental genotypes tested.

seedling were different from the *P. palustris* receptor sequences. The chloroplast DNA of *P. palustris* is quite clearly different from *P. pratensis* (Patterson et al., 2005); thus, we concluded this transgenic seedling was a contaminant from BR99-1033 seed. The chloroplast DNA of two transgenic *P. arachnifera* offspring contained the AY589107 *ndhF* and DQ389141 *rps16-trnK* sequences, which are unique to the *P. arachnifera* receptor plants (i.e.,

different from all other *Poa* species tested). As mentioned above, the third transgenic *P. arachnifera* offspring died before DNA was collected. The chloroplast DNA of all seven transgenic *P. interior* offspring contained the *ndhF*-2 allele shared only by *P. interior*, *P. palustris,* and *P. nemoralis* and the DQ389137 *rps16-trnK* sequence found only in the *P. interior* accession (i.e., different from all other *Poa* species tested). The chloroplast DNA sequences of 21 transgenic *P. secunda* × *P. pratensis* PI 578808 offspring and 11 transgenic *P. secunda* × *P. pratensis* PI 578818 offspring contained the *ndhF*-2 allele shared only by the respective maternal receptor parents and the *P. secunda* PI 578851 genotypes. The PCR or sequencing reactions failed for six transgenic *P. secunda* × *P. pratensis* PI 578808 offspring and one transgenic *P. secunda* × *P. pratensis* PI 578818 offspring, but otherwise all putative interspecific hybrids were confirmed (Table 2). These data also confirm the maternal *P. secunda* lineage of the *P. secunda* × *P. pratensis* hybrid accessions (PI 578808 and PI 578818) and distinguish these genotypes from several other *P. secunda* sequences (AY589111 and AY589112).

All *P. pratensis* varieties tested shared the same chloroplast *ndhF*-1 DNA sequence. Thus, the *ndhF* marker could not distinguish the paternal (BR99-1033) and maternal parents of the intraspecific hybrids. The marker does, however, effectively confirm the maternal *P. pratensis* identity of the intraspecific hybrids (i.e., they are not interspecific hybrid seed contaminations). The chloroplast *rps16-trnK* DNA sequences from most of the 112 intraspecific hybrids (*P. pratensis*) were identical to the nontransgenic maternal receptor accessions but different from the transgenic BR99-1033 genotype (Table 2).

The experimental BR99-1033 transgenic variety showed relatively uniform appearance, uniform DNA profiles (results not shown), and did not segregate for glyphosate tolerance. Thus, we deduce that it is fixed for the chloroplast *rps16-trnK*-3 allele, which was different from all other bluegrasses analyzed except one transgenic Midnight offspring, one transgenic Fairfax offspring, and the Fairfax receptor genotype. The chloroplast *rps16-trnK* DNA sequences from nine of the other 12 transgenic Midnight offspring contained the *rps16-trnK*-2 allele characteristic of the Midnight receptor plants. The other two transgenic offspring of Midnight carry the *rps16-trnK*-1 allele characteristic of the Rutgers receptor genotype. Thus, *rps16-trn* sequences from 11 of the 12 transgenic Midnight offspring are different from BR99-1033 and most contain the *rps16-trnK*-2 allele characteristic of the Midnight receptor plants. Likewise, the chloroplast *rps16-trnK* DNA sequences from 79 of the 80 transgenic Rutgers offspring contain the *rps16-trnK*-1 allele of the Rutgers receptor genotype. The chloroplast *rps16-trnK* DNA sequence from one other transgenic Rutgers offspring contained the *rps16-trnK*-2 allele present in two Midnight receptor genotypes and most of the transgenic Midnight offspring. The chloroplast *rps16-trnK* DNA sequences from 14 of the 16 transgenic Kenblue offspring and five of the seven transgenic *P. pratensis* subsp. *angustifolia* PI 317504 offspring contain the *rps16-trnK*-4 allele, which is shared only by Kenblue and *P. pratensis* subsp. *angustifolia* PI 317504 receptor genotypes. As for the other two transgenic *P. pratensis* subsp. *angustifolia* PI 317504 offspring we detected one unique *rps16-trnK* sequence (DQ389140), albeit similar to other *P. pratensis* sequences, but not found in any other plant. We did not successfully sequence the other putative hybrid.

With one exception (*P. palustris*), we effectively confirmed most of the putative hybrids and have reasonable justification to assume correct identity for unconfirmed hybrids. With the single exception of Fairfax, which was not distinguishable from BR99-1033, most putative hybrids within each receptor genotype (Table 2) were properly confirmed.

Distance and Direction of Gene Flow

In 2002, we observed the highest level of gene flow to other *Poa* entries in the center plot (0-m distance). The number of hybrids detected decreased at the 13-m distance and far fewer at 53 m (Table 3). Four of the six hybrids observed at the 53-m distance were in the NE direction from one plant. Only one hybrid was detected at 53 m from a vector other than to the NE. (Table 3). Results in 2003 were similar, but gene flow was much lower (Table 4). The numbers of seedlings evaluated were decreased because of billbug insect (*Sphenophorous* spp.) damage on many of the species in late summer 2002 and poor seed set conditions due to hot, dry weather during anthesis in 2003. Hybridization rates in the center plot entries (0 m) was 0.38% for *P. pratensis* and two hybrids out of 15 seedlings (13.3%) from *P. arachnifera*. More seedlings were obtained from *P. arachnifera* in 2003, as the plants were significantly larger because of an additional year growth. Overall, hybridization at 13 and 53 m in 2003 was again lower than in the center plot (Table 4).

Although the generally low number of hybrids detected limited our ability to correlate wind direction and hybridization rates, most hybrids appeared to occur in plots downwind of the BR99-1033 in 2002 and 2003. However, pollen movement and resulting gene flow was not limited to the prevailing downwind directions since hybrids were detected throughout the layout of the experiment (Tables 3 and 4). Wind direction significantly influences the direction of pollen flow, but wind turbulence, wind irregularity, and wind speed are also determinants of pollen and gene flow (Giddings et al., 1997a, 1997b). Our experiment used approximately 500 pollen source plants, compared with 40 plants Giddings et al. (1997a, 1997b) and 286 in work by Wipff and Fricker (2001). As the size of the pollen source increases, pollen and potential gene flow becomes more unpredictable in terms of direction (Giddings, 2000).

Because so few hybrids were detected, we were unable to effectively model gene flow over distance and direction. When compared with the models used most effectively in other gene flow reports (Wipff and Fricker, 2001; Cunliffe et al., 2004), the fit of our data was poor, with an r^2 value of 0.22 in an exponential decay model. Although pollen can move long distances, even hundreds of miles, the majority of gene flow occurs over very short distances (Gleaves, 1973). In *Festuca pratensis* Huds., a predominantly out crossing species, the ability of intra-

Table 3. 2002 Hybrid summary for species and accession in receptor plots where hybrid progeny were detected†.

Direction	Distance	Species/ accession	Seedlings		
			Total no.	No. hybrids	% hybrids
	m				
Center	0	*Poa* hybrid‡	848	17	2.00
		PI 578808	430	12	2.79
		PI 578818	418	5	1.20
Center	0	*P. interior*	788	5	0.63
Center	0	*P. pratensis*	2638	83	3.15
		PI 317504	–	–	–
		Coventry	55	0	0
		Fairfax	84	1	1.19
		Kenblue	79	6	7.60
		Midnight	2002	10	0.05
		Rutgers	418	66	15.79
NE	13	*Poa* hybrid	799	11	1.38
		PI 578808	557	10	1.80
		PI 578818	242	1	0.41
NE	13	*P. interior*	390	0	0
NE	13	*P. pratensis*	2727	4	0.15
		PI 317504	–	–	–
		Coventry	644	0	0
		Fairfax	478	0	0
		Kenblue	337	1	0.30
		Midnight	320	0	0
		Rutgers	948	3	0.32
NE	53	*Poa* hybrid	634	4	0.63
		PI 578808	290	4	1.38
		PI 578818	344	0	0
NE	53	*P. interior*	805	1	0.12
NE	53	*P. pratensis*	3757	1	0.03
		PI 317504	–	–	–
		Coventry	783	0	0
		Fairfax	821	0	0
		Kenblue	642	0	0
		Midnight	712	0	0
		Rutgers	799	1	0.13
NW	13	*Poa* hybrid	300	5	1.67
		PI 578808	35	0	0
		PI 578818	265	5	1.89
NW	13	*P. interior*	520	2	0.38
NW	13	*P. pratensis*	1674	2	0.12
		PI 317504	–	–	–
		Coventry	76	0	0
		Fairfax	183	0	0
		Kenblue	930	2	0.22
		Midnight	–	–	–
		Rutgers	485	0	0
W	13	*P. pratensis*	4565	1	0.02
		PI 317504	–	–	–
		Coventry	995	0	0
		Fairfax	538	0	0
		Kenblue	546	1	0.18
		Midnight	1829	0	0
		Rutgers	657	0	0
W	53	*Poa* hybrid	2613	1	0.04
		PI 578808	2498	0	0
		PI 578818	115	1	0.87
SW	13	*P. pratensis*	7515	4	0.05
		PI 317504	–	–	–
		Coventry	1609	0	0
		Fairfax	325	0	0
		Kenblue	1699	3	0.18
		Midnight	2850	0	0
		Rutgers	1032	1	0.10
SE	13	*P. pratensis*	2580	2	0.08
		PI 317504	–	–	–
		Coventry	118	0	0
		Fairfax	81	0	0
		Kenblue	149	1	0.67
		Midnight	1340	0	0
		Rutgers	892	1	0.11

† List of all entries that showed hybrids throughout the experiment.
‡ *Poa pratensis* × *P. secunda* hybrid.

Table 4. 2003 hybrid summary for *P. hybrid*, *P. interior*, and *P. pratensis*.†

Direction	Distance	Species/ accession	Seedlings		
			Total no.	No. hybrids	% hybrids
	m				
Center	0	*Poa* hybrid‡	179	0	0
		PI 578808	121	0	0
		PI 578818	58	0	0
Center	0	*P. interior*	770	0	0
Center	0	*P. pratensis*	2350	13	0.55
		PI 317504	53	4	7.55
		Coventry	196	0	0
		Fairfax	151	0	0
		Kenblue	321	0	0
		Midnight	1076	1	0.09
		Rutgers	553	8	1.45
Center	0	*P. arachnifera*	15	2	13.33
NE	53	*Poa* hybrid	2107	0	0
		PI 578808	1342	0	0
		PI 578818	765	0	0
NE	13	*Poa* hybrid	1087	0	0
		PI 578808	921	0	0
		PI 578818	166	0	0
NE	13	*P. interior*	1522	0	0
NE	13	*P. pratensis*	3741	1	0.03
		PI 317504	669	1	0.15
		Coventry	86	0	0
		Fairfax	26	0	0
		Kenblue	1224	0	0
		Midnight	675	0	0
		Rutgers	1061	0	0
NE	13	*P. arachnifera*	2	0	0
NW	13	*Poa* hybrid	224	0	0
		PI 578808	153	0	0
		PI 578818	71	0	0
NW	13	*P. pratensis*	787	2	0.25
		PI 317504	148	1	0.68
		Coventry	5	0	0
		Fairfax	9	0	0
		Kenblue	230	1	0.44
		Midnight	395	0	0
		Rutgers	–	–	–
NW	53	*Poa* hybrid	734	1	0.14
		PI 578808	361	1	0.28
		PI 578818	373	0	0
NW	53	*P. interior*	420	0	0
NW	53	*P. pratensis*	3145	0	0
		PI 317504	52	0	0
		Coventry	5	0	0
		Fairfax	28	0	0
		Kenblue	778	0	0
		Midnight	642	0	0
		Rutgers	1640	0	0
NW	53	*P. arachnifera*	3	0	0
SE	13	*Poa* hybrid	218	0	0
		PI 578808	175	0	0
		PI 578818	43	0	0
SE	13	*P. interior*	426	0	0
SE	13	*P. pratensis*	872	2	0.23
		PI 317504	68	0	0
		Coventry	7	0	0
		Fairfax	5	0	0
		Kenblue	530	1	0.19
		Midnight	141	1	0.71
		Rutgers	121	0	0
SE	13	*P. arachnifera*	15	1	6.67
SW	13	*Poa* hybrid	795	0	0
		PI 578808	664	0	0
		PI 578818	131	0	0
SW	13	*P. interior*	1816	0	0
SW	13	*P. pratensis*	2529	1	0.04
		PI 317504	56	1	1.80
		Coventry	832	0	0
		Fairfax	–	–	–
		Kenblue	1487	0	0
		Midnight	–	–	–
		Rutgers	154	0	0
SW	13	*P. arachnifera*	8	0	0

Continued on next page.

Table 4. Continued.

Direction	Distance	Species/ accession	Seedlings		
			Total no.	No. hybrids	% hybrids
	m				
W	53	*Poa* hybrid	1785	0	0
		PI 578808	1282	0	0
		PI 578818	503	0	0
W	53	*P. interior*	423	0	00
W	53	*P. pratensis*	1167	1	0.09
		PI 317504	372	0	0
		Coventry	75	0	0
		Fairfax	–	–	–
		Kenblue	437	0	0
		Midnight	–	–	–
		Rutgers	283	1	0.35
W	53	*P. arachnifera*	1	0	0

† List of all entries that showed hybrids throughout the experiment.
‡ *Poa pratensis* × *P. secunda* hybrid.

specific hybrids to occur at distances from a pollen source were heavily dependent on the density of the potential receptor plants (Rognli et al., 2000). Isolated plants are more likely to hybridize with distant pollen sources than communities of plants (Gleaves, 1973; Rognli et al., 2000). Increasing the number of plants between the pollen source and receptor plots in our experiment may have resulted in more hybrids produced and possibly better gene flow predictions, but the additional plants could further reduce the fit to a exponential decay model through greater pollen competition and pollen flow variability.

CONCLUSIONS

These results demonstrate that apomictic *P. pratensis* turfgrass cultivars can produce viable pollen and create hybrids in field conditions with other *Poa* species. However, amount of gene flow was low, especially at 15 m and beyond. Apomixis of the receptor plants and pollen competition from surrounding *Poa* may significantly influence hybrid occurrence and gene flow.

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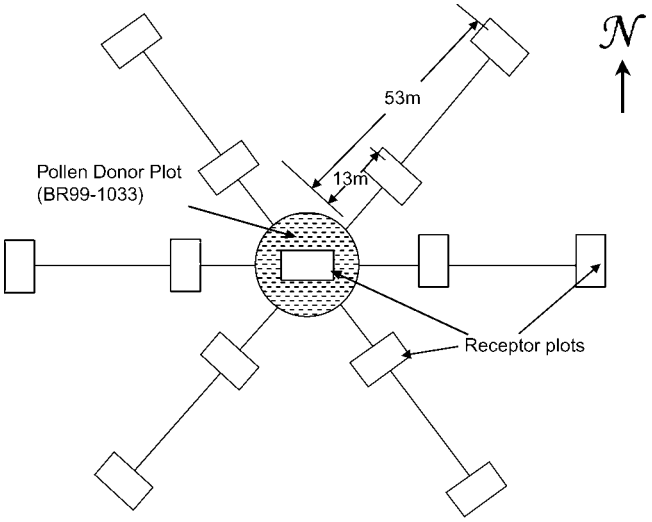

Fig. S1. Diagram of the overall field plot area with spacing between pollen donor plot at center and receptor plots containing the *Poa* species entries.

Table S1. PCR primer sequences used to verify putative transgenic *Poa* hybrids.

Primer	Sequence 5′–3′
35S-1	**GCTCCTACAAATGCCATC**
35S-2	**GATAGTGGGATTGTGCGTCA**
NOS-1	**GAATCCTGTTGCCGGTCTTG**
NOS-3	**TTATCCTAGTTTGCGCGCTA**
***ndhF* 1318**	**GGATTAACYGCATTTTATATGTTTCG**
***ndhF* 2110R**	**CCCCCTAYATATTTGATACCTTCTCC**
***trnK* 5′r**	**TACTCTACCRTTGAGTTAGCAAC**
***rps16*-4547**	**AAAGGKGCTCAACCTACARGAAC**